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Optimization of the aqueous enzymatic extraction of pine kernel oil by response surface methodology

Yang Li[a,b], Lianzhou Jiang[a,b,]a*, Xiaonan Sui[a,b], Shengnan Wang[a,b]

[a]*Food Science College, Northeast Agricultural University, Harbin 150030,China*
[b]*National Institute of Soybean Engineering Technology, Harbin 150030,China*

Abstract

Alcalase endo-protease enzyme was selected in this experiment to extract oil from pine kernel. The response surface analysis method was employed to optimize the parameters in the experiment. The optimum parameters are as follows: enzyme additive amount 1.97%, hydrolysis time 3.0h, hydrolysis temperature 51 ℃, materials to water rate 1:5, pH 8.4 and total oil extraction rate 89.12%. Factor contribution rate obtained from the F-test are as follows: $x_2 > x_1 > x_4 > x_3 > x_5$. The fatty acid contents of pine kernel oil are as follows: palmitc acid 3.89%, oleic acid 19.44%, linoleic acid 50.09%, linolenic acid 0.58% and stearic acid 1.53%.

Key words: Aqueous enzymatic extraction; Alcalase; Pine seed oil; Response surface; Fatty acid

1. Introduction

Pine kernel is the seed of Pinus koraiensis, which rich in protein, dietary fiber, carbohydrates and other nutrients. Besides it has higher concentration of containsα-linolenic acid and other essential polyunsaturated fatty acids [1]. Pine oil is extracted from natural red pine, which is the ideal green oil

* Corresponding author. Tel.: +86-13904652669; fax: +86-0451-55190716.
E-mail address: jlzname@163.com. Research Grant Number: GA09B401-6

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without pollution. Its amount is large. With the rising of the pursuit of natural food, pine kernel-related research and product development of functional food research have become a new bright spot, which not only increase the edible oil varieties and adjust the structure of food and nutrition in people's diet, but also improve economic benefits; therefore pine oil has broaden development prospects [2].

Aqueous enzymatic extraction has been extensively studied. It has received much interest, and viewed as an alternative method to extract oil from oil-bearing seeds [3]. Not only this green technology is beneficial to people's health but also is environmental friendly.

In this study, alkaline protease "alcalase" was used in the extraction of pine kernel oil. The single factor experiments and response surface methods were employed to optimize the parameters in oil extraction process. Meanwhile, the five sorts of fatty acids in kernel oil were determined which provide theoretical guidance of aqueous enzymatic extraction of pine oil.

2. Methods

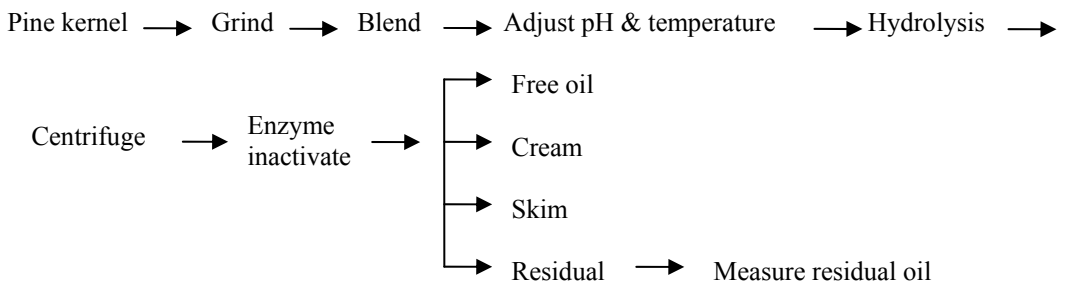
2.1. Materials

Peeling pine nuts, produced in Changbai Mountain in China. The main ingredient in pine kernel shows in Tab.1. Alcalase alkaline endo-protease (activity 1.2×105U/mL; Novo Company) was employed in this experiment. Autosystem GC/Q-Mass 910 gas chromatograph (P.E. USA Inc.), LDZ5-2-type low-speed desktop centrifuge, DU800 UV spectrophotometer, electronic balance, F2102 type of plant specimen grinder, electric heated water bath, precision electric mixer, soxhlet extractor and electro-thermostatic blast oven were used in this experiment.

Table.1 Main components of pine seed oil

Ingredient	Crude protein	Crude fat	Water	Ash
Content (%)	16.6	63.8	4.2	2.4

2.2. Procedure



2.3. Single factor experiments

(1) The parameters of the effect of enzyme additive amount on total oil extraction single-factor experiment are as follows: materials to water rate 1:6, hydrolysis temperature 50 ºC, hydrolysis time 4h and pH 9. The selected enzyme additive amounts are 0.5%, 1%, 1.5%, 2%, 2.5%, 3% and 3.5%.

(2) The parameters of the effect of temperature on total oil extraction single-factor experiment are as follows: materials to water ratio 1:6, hydrolysis time 4h, pH 9 and enzyme additive amount 2%. The chosen reaction temperature are 40 ºC, 45 ºC, 50 ºC, 55 ºC, 60 ºC and 65 ºC.

(3) The parameters of the effect of time on total oil extraction single-factor experiment are as follows: materials to water ratio 1:6, hydrolysis temperature 50 ºC, pH 9 and enzyme additive amount 2%. The chosen hydrolysis times are 0.5 h, 1.5 h, 2h, 2.5 h, 3.5 h, 4.5 h and 5.5 h.

(4) The parameters of the effect of materials to water ratio on total oil extraction single-factor experiment are as follows: hydrolysis temperature 50 ºC, pH 9 and enzyme additive amount 2%. The chosen materials to water ratios are 1:2, 1:4, 1:6, 1:8, 1:10 and 1:12.

(5) The parameters of the effect of pH on total oil extraction single-factor experiment are as follows: materials to water ratio 1:6, hydrolysis temperature 50 ºC and enzyme additive amount 2%. The chosen pH is 7, 8, 9, 10, 11 and 12.

2.4. Response surface method analysis

The range of level values of each factors were determined based on the single-factor experiments parameters. The response surface method was employed to analysis the effects of each factors on total oil extraction rate. Five factors (enzyme additive amount, hydrolysis temperature, hydrolysis time, materials to water ratio and pH) were selected as independent variable and total oil rate was chosen as variable.

Determination of water: according to GB 5009.3-2010

Determination of fat: according to GB/T 14772-2008

Determination of crude protein: according to GB 5009.5-2010

Determination of ash: according to GB/T 5505-2008

Weigh 30g pine nuts grinding powder, and then mixed with distilled water according to a certain materials to water ratio. Then, transfer the mixture into water bath with continuous stirring and adjust the mixture pH using NaOH. Alkaline endo-protease (Alcalase) was added into the mixture and keep the hydrolysis temperature during the enzymatic hydrolysis process. Then, inactivate the enzyme by increasing temperature to 100 ºC for 5 min and centrifuge the mixture. After then, absorb the oil in upper layer and dry the residual materials. SAS 9.2 (SAS Institute Inc., USA) software was used to analysis the date of the experiment [4]. Equation 1 is used to calculate the total oil extraction rate of Pine kernel:

$$\text{Total oil extraction rate (\%)} = \frac{\text{Total oil in Pine Kernel} - \text{Oil in residual materials}}{\text{Total oil in Pine Kernel}} \times 100 \quad (1)$$

3. Results and discussion

3.1. The analysis of single factor experiment

3.1.1The effect of enzyme additive amount on oil extraction rate

Fig.1 shows that the oil extraction rate is increased with the increasing additive amount of enzyme. The oil extraction rate reaches its highest point when the additive amount of enzyme is 2%. However, the

oil extraction rate keeps stably when the additive amount beyond 2%. The reason of this trend is that the hydrolysis process is more adequate with the increasing of enzyme amount. The enzyme penetrates into the liposome membrane movement and the decomposition function of enzyme to lipopolysaccharide and lipoprotein of great benefit to the oil release [5]. The optimum enzyme additive amount is 2%.

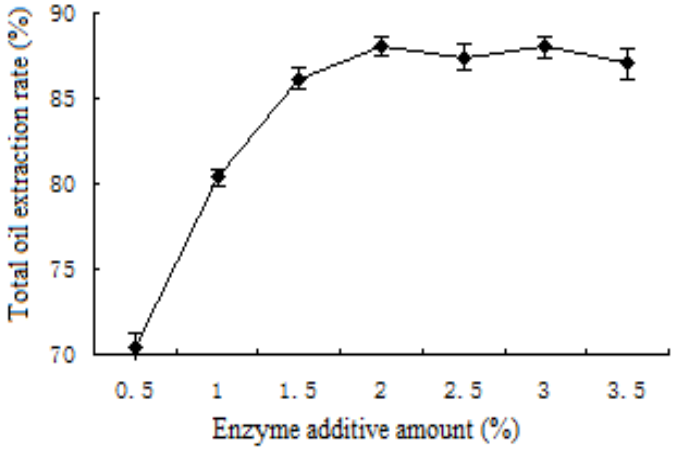


Fig.1 Effects of enzyme additive amount on oil extraction rate

3.1.2 The effect of hydrolysis temperature on oil extraction rate

Fig.2 indicates that the oil extraction rate is increased with the increasing temperature (under 50 ºC) and decreased with the increasing temperature (beyond 50 ºC). The reason is that the suitable temperature of enzyme is below 50 ºC and the higher temperature inactivate enzyme (beyond 50 ºC). The suitable temperature is 50 ºC.

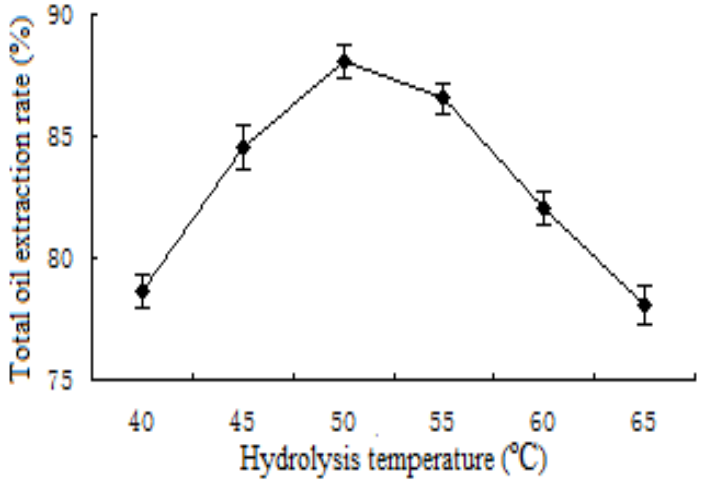


Fig.2 Effects of hydrolysis temperature on oil extraction rate

3.1.3 The effect of hydrolysis time on oil extraction rate

Fig. 3 shows that the oil extraction rate increase sharply when the hydrolysis time under 2.5h and keep stable with the increasing of time, which illustrates that the interaction between enzyme and substrate is totally complete. Therefore, the suitable hydrolysis time is 2.0~3.5h.

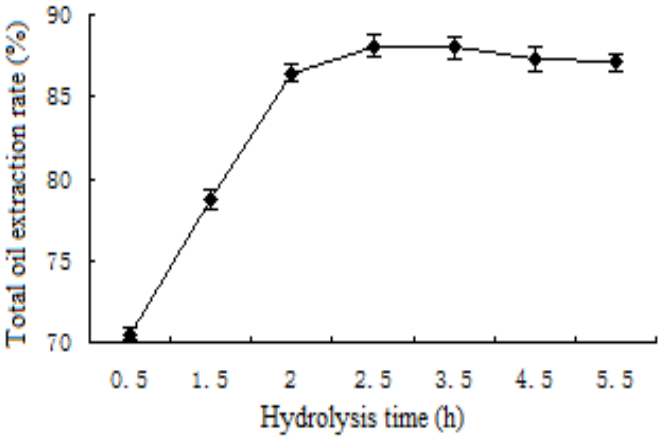


Fig.3 Effects of hydrolysis time on oil extraction rate

3.1.4 The effect of materials to water rate on oil extraction rate

Fig.4 illustrates that the oil extraction rate is increased first and then decreased gradually with the decrease of materials to water rate. The oil extraction rate reaches its peak at materials to water rate 1:6. From previous papers, we know that water plays a key role in hydrolysis process, however, overwhelming water decrease the concentration of enzyme and substrate which decrease the opportunity of collision between enzyme and substrate at molecular level [7]. Then, the suitable materials to water rate is 1:6.

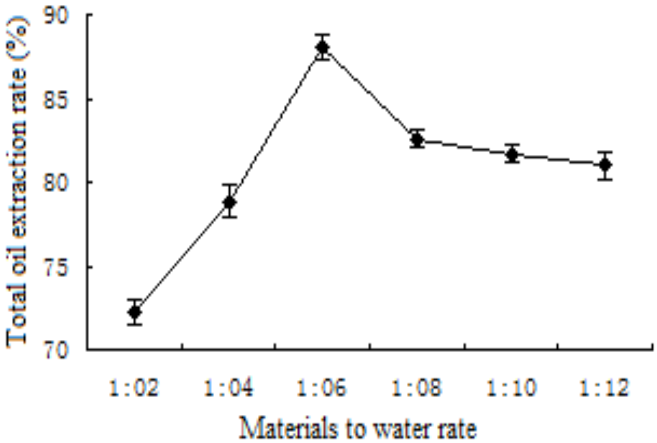


Fig.4 Effects of ratio of material to water on oil extraction rate

3.1.5 The effect of pH on oil extraction rate

Fig.5 shows that the oil extraction rate is gradually increased with the increase of pH. The highest oil extraction rate is reached when the pH is 9, and then the rate fell along with the decrease of pH. The main reason of this phenomenon is that the suitable pH of Alcalase is 9. The enzyme is at its highest activity at pH 9 and more oil extracted [8]. The suitable pH is 9.

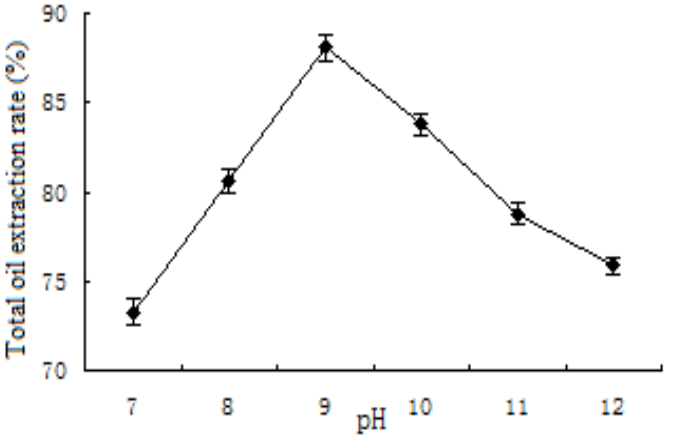


Fig.5 Effects of pH value on oil extraction rate

3.2. Response surface analysis

The range of each factor's level is determined based on the data of single factor experiment. The response surface analysis method is employed to design and optimize the parameters in the experiment. The independent variables are as follows: enzyme additive amount (x1), hydrolysis temperature (x2), hydrolysis time (x3), materials to water rate (x4) and pH (x5). The response value is total oil extraction rate. Tab.2 is the level coding and Tab.3 is the design and result of this experiment. Experimental No.1-26 is the factorial and 27-36 is center trial used to estimate the experimental error.

Table.2 Encode table of factors and levels

Code	Factor				
	Enzyme additive amount x_1 (%)	Hydrolysis temperature x_2 (℃)	Hydrolysis time x_3 (h)	Materials to water rate x_4	pH x_5
-2	1	40	2.0	1：4	8
-1	1.5	45	2.4	1：5	8.5
0	2.0	50	2.8.	1：6	9.0
1	2.5	55	3.2	1：7	9.5
2	3	60	3.6	1：8	10.0

Table.3 Design and results of response surface analysis

Run	Enzyme additive amount x_1 (%)	Hydrolysis temperature x_2 (℃)	Hydrolysis time x_3 (h)	Materials to water rate x_4	pH x_5	Total oil extraction rate y(%)
1	-1	-1	-1	-1	1	74.88
2	-1	-1	-1	1	-1	72.98
3	-1	-1	1	-1	-1	84.88
4	-1	-1	1	1	1	72.44
5	-1	1	-1	-1	-1	88.57
6	-1	1	-1	1	1	85.64
7	-1	1	1	-1	1	82.86
8	-1	1	1	1	-1	80.44
9	1	-1	-1	-1	-1	82.06
10	1	-1	-1	1	1	80.24
11	1	-1	1	-1	1	82.43
12	1	-1	1	1	-1	80.86
13	1	1	-1	-1	1	87.56
14	1	1	-1	1	-1	83.55
15	1	1	1	-1	-1	83.64
16	1	1	1	1	1	87.76
17	-2	0	0	0	0	70.98
18	2	0	0	0	0	88.88
19	0	-2	0	0	0	75.38
20	0	2	0	0	0	87.38
21	0	0	-2	0	0	82.44
22	0	0	2	0	0	87.66
23	0	0	0	-2	0	81.46
24	0	0	0	2	0	81.24
25	0	0	0	0	-2	85.96
26	0	0	0	0	2	87.46
27	0	0	0	0	0	89.88
28	0	0	0	0	0	90.01
29	0	0	0	0	0	89.58
30	0	0	0	0	0	86.48
31	0	0	0	0	0	89.55
32	0	0	0	0	0	90.78

The response surface regression model Eq.2 is established by SAS 9.2.

$$y=89.03671+1.826006x_1+3.052083x_2+0.427917x_3-0.975417x_4-0.007083x_5-3.125412x_1^2-0.963125x_1x_2+0.170625x_1x_3+1.025625x_1x_4+1.183125x_1x_5-1.676296x_2^2-1.316875x_2x_3+0.780625x_2x_4+1.150625x_2x_5-0.758796x_3^2-0.103125x_3x_4-0.343125x_3x_5-1.683796x_4^2+1.229375x_4x_5-0.343796x_5^2 \quad (2)$$

Tab.4 shows the results of regression and variance analysis and Fig.6 illustrates the results of dimension reduction analysis. Tab.5 is the results of response surface optimization and Fig.7 is the response surface analysis of significant effective interaction items.

From Tab.4, the linear relationship between dependent variable and independent variables is significant, the regression model is significant ($p < 0.001$), lack of fit is insignificant, R2= 94.85% and R2Adj=85.48%, which indicate that the model fits well with the experimental data and the model can be used to analysis and predict the results of enzymatic extraction of pine kernel oil. Factor contribution rate obtained from the F-test are as follows: x2 > x1 > x4 > x3 > x5 (hydrolysis temperature > enzyme additive amount > materials to water ratio > pH > hydrolysis time).

Table.4 Results of regression and variance analysis.

Various	Degrees of freedom	Sum of squares	Mean square	F	Pr>F
x_1	1	56.56809	56.56809	12.85879	0.0043
x_2	1	223.5651	223.5651	50.81976	<.0001
x_3	1	4.394704	4.394704	0.998983	0.3390
x_4	1	22.8345	22.8345	5.190631	0.0437
x_5	1	0.001204	0.001204	0.000274	0.9871
x_1^2	1	164.3061	164.3061	37.34928	<.0001
x_1x_2	1	14.84176	14.84176	3.373758	0.0934
x_1x_3	1	0.465806	0.465806	0.105885	0.7510
x_1x_4	1	16.83051	16.83051	3.825831	0.0763
x_1x_5	1	22.39656	22.39656	5.091079	0.0454
x_2^2	1	81.92616	81.92616	18.62307	0.0012
x_2x_3	1	27.74656	27.74656	6.307216	0.0289
x_2x_4	1	9.750006	9.750006	2.216325	0.1647
x_2x_5	1	21.18301	21.18301	4.815221	0.0506
x_3^2	1	16.78692	16.78692	3.815924	0.0767
x_3x_4	1	0.170156	0.170156	0.038679	0.8477

x_3x_5	1	1.883756	1.883756	0.428207	0.5263
x_4^2	1	82.6609	82.6609	18.79008	0.0012
x_4x_5	1	24.18181	24.18181	5.496894	0.0389
x_5^2	1	3.446056	3.446056	0.783341	0.3951
Regression	20	890.5082	44.52541	10.12131	0.0002
Residual	11	48.39094	4.399177		
Lack of fit	5	37.09086	7.418171	3.938822	0.0627
Sum	31	938.8991			

Fig.6 shows the law of the effect of enzymatic parameters on total protein extraction rate. The total oil extraction rate increases with the increasing of enzyme additive amount. And the rate increases first and decreases with the rising hydrolysis temperature, which mainly due to that the effect of alkaline protease is better in its suitable temperature. Total oil extraction rate increases with the prolonging hydrolysis time; however, the rate falls after a certain time. Total oil extraction rate increases with the increasing of materials to water rate, while the continuous increasing of materials to water rate will dilution the concentrations of enzyme and substrate by decreasing the collision probabilities of enzyme and substrate at molecules level. Therefore, the total oil extraction rate decreases. The total oil extraction rate increases first and then decreases with the rise of pH.

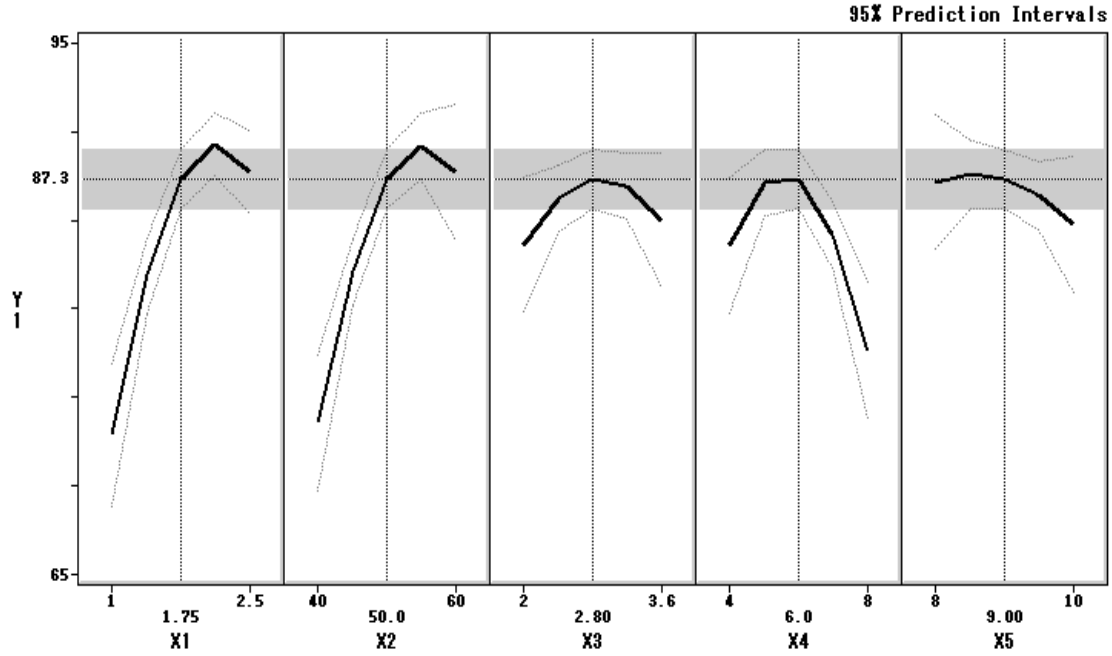
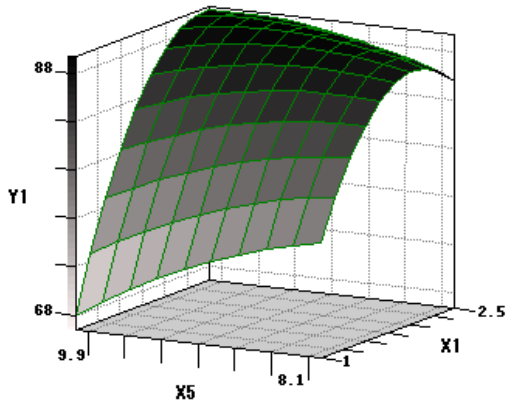


Fig.6 Dimension reduction analysis of effect of different hydrolysis parameters on extraction rate of pine kernel oil

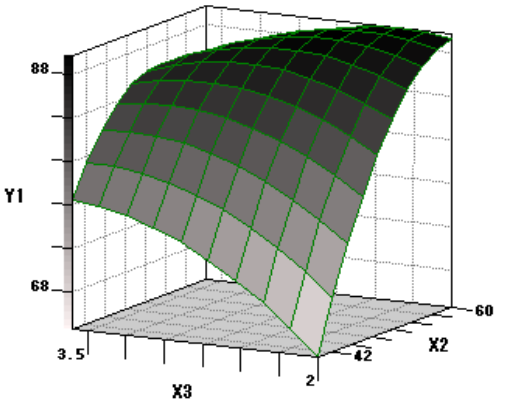
Tab.5 shows the optimum parameters obtained from response surface analysis. The optimum parameters are as follows: enzyme additive amount 1.97%, hydrolysis time 3.0h, hydrolysis temperature 51 ºC, materials to water rate 1:5, pH 8.4 and total oil extraction rate 89.75±1.01.

Table.5 Optimum results of response surface

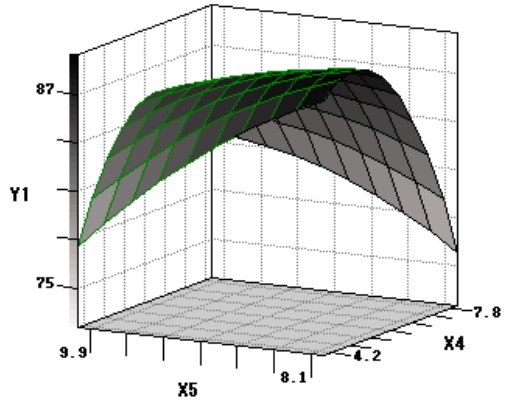
Factor	Level value	Actual value	Order	Total oil extraction rate
x_1	0.05996	1.9700	1.97	89.75±1.01
x_2	0.22952	51.1476	51	
x_3	0.37952	2.9518	3.0	
x_4	0.68150	5.3185	1:5	
x_5	1.13728	8.4314	8.4	



A: $y=f(x_1, x_5)$



B: $y=f(x_2, x_3)$



C: $y=f(x_4, x_5)$

Fig.7 Response surface analysis of significant effective interaction items of different hydrolysis parameters on extraction rate of pine kernel oil

3.3. Verification experiment

Three parallel experiments were taken under the best conditions: enzyme additive amount 1.97%, hydrolysis time 3.0h, hydrolysis temperature 51 ºC, materials to water rate 1:5 and pH 8.4. The average result of the three parallel experiments is 89.12% which indicates that the response value fits well with the regression predict value and the model can predict the actual condition of pin kernel oil extraction.

3.4. Pine kernel oil fatty acid analysis

Gas chromatography [9] was used to identify components in the pine kernel oil. The fatty acid contents of pine kernel oil are as follows: palmitc acid 3.89%, oleic acid 19.44%, linoleic acid 50.09%, linolenic acid 0.58% and stearic acid 1.53%.

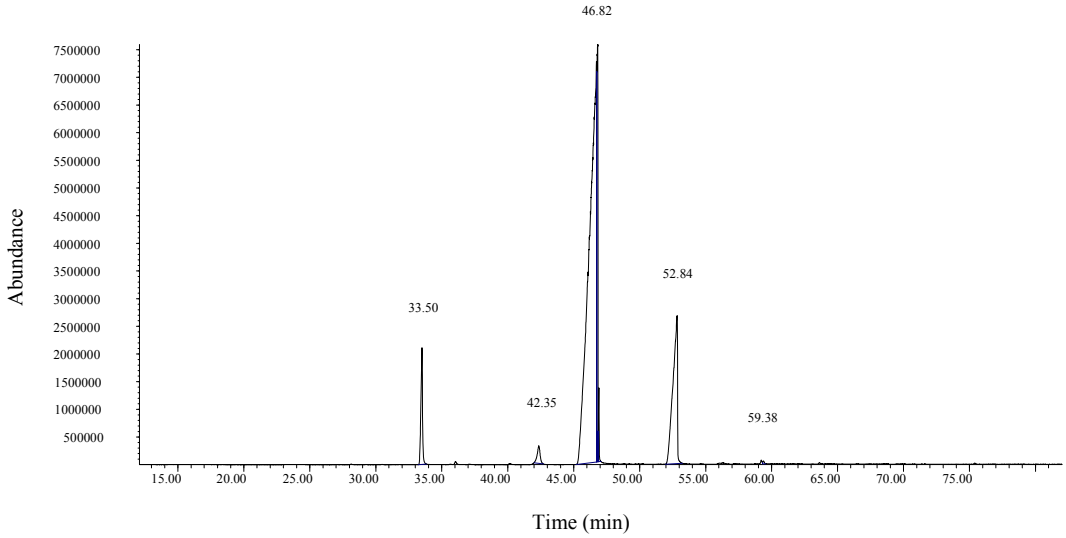


Fig.8 Gas chromatography analysis of pine kernel oil

4. Conclusion

Alcalase endo-protease enzyme was selected in this experiment to extract oil from pine kernel. The response surface analysis method was employed to optimize the parameters in the experiment. The optimum parameters are as follows: enzyme additive amount 1.97%, hydrolysis time 3.0h, hydrolysis temperature 51 ºC, materials to water rate 1:5, pH 8.4 and total oil extraction rate 89.12%. Factor contribution rate obtained from the F-test are as follows: x2 > x1 > x4 > x3 > x5. The fatty acid contents of pine kernel oil are as follows: palmitc acid 3.89%, oleic acid 19.44%, linoleic acid 50.09%, linolenic acid 0.58% and stearic acid 1.53%.

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